A NON-INVASIVE METHOD FOR TRACING ORGANIC FISH

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NEINVAZIVNA METODA ZA UTVRĐIVANJE ORGANSKI GAJENE RIBE

Apstrakt

U poslednjih nekoliko godina, prisutne su drugačije tendencije u akvakulturi, koje pre svega imaju za cilj plasiranje novih proizvoda, od kojih je jedan i riba gajena u organskoj akvakulturi. Razlikovanje ribe gajene u organskoj akvakulturi od one gajene na konvencionalni način je teško, ali se razlika može napraviti preko njenog izgleda. U ovom eksperimentu brancin je hranjen konvencionalnom i organskom hranom i u toku gajenja, pravljene su fotografije primeraka. Nakon kalibracije boje, određene su merne tačke na svakoj fotografiji, a nakon toga su geometrijskim i morfometrijskim metodama dobijene RGB matrice. Tako dobijena matrica (195x135,225) je prvobitno analizirana korišćenjem 50-50 MANOVA metode, a nakon toga su urađeni diskriminantna analiza i na kraju dendrogram. Svi uzorci su klasifikovani korišćenjem tri diskriminantna modela. Tako je dendrogram sa ukupno 9 različitih klasa pokazao da se ribe koje su gajene u organskoj akvakulturi slične ribama uzorkovanim iz prirodnih populacija. Rezultati su pokazali i da dve grupe riba, hranjenih različitim komercijalnim hranama u ovom eksperimentu, jedne u organskoj, a druge u konvencionalnoj akvakulturi mogu biti prepoznate po boji njihovog tela. Šta više, što duže vremena ribe provedu u jednom od ova dva načina gajenja, to se boja njihovog tela više razlikuje. Deo tela koji pokazuje najveće promene u boji jeste glava, koja je značajno svetlije boje u grupi riba gajenih u organskoj akvakulturi. Tako je dokazano da analiza boje tela može biti iskorišćena za razlikovanje riba koje su gajene u drugačijim uslovima korišćenjem različitih protokola i načina gajenja. Međutim, ovaj zaključak se može primeniti samo na ove, konkretne

podatke, ne može biti generalizovan i ne može se primeniti na sve ribe gajene u organskoj akvakulturi.

Ključne reči: Dicentrarchus labrax, brancin, akvakultura, organska proizvodnja, morfologija

Keywords: Dicentrarchus labrax, sea bass, aquaculture, organic farming, morphology

INTRODUCTION

In the last 20 years, fish aquaculture companies have tried to find ways of diversification at every level of the production pipeline, from the start - by introducing alternative species or developing niche and "minor" productions (Bianchini and Palmegiano, 1994) up to the consumer end, e.g. offering fillets, fish patties, ready-to-cook preparations, etc. (Bianchini et al., 2010). One form of product diversification could also be implemented during the raising phase by employing methods of organic farming (Cataudella et al., 2001).

Organic fish farming is still a small fraction of the aquaculture total output (less than 1%), but nevertheless it is worth more than 50 million euros annually (IFOAM, 2010).

Organic certification and labeling (IFOAM, 2010) goes together; in fact, without long and expensive analyses, it would otherwise be impossible to discriminate a fish grown organically from another raised with conventional practices. On the other hand, appearance is used throughout all production stages, for sorting by size (Costa *et al*., 2013a) or for defects (Bianchini *et al*., 1994), but also as a primary mean for judging the quality of individual units of product, and might be useful in tracing the fish origins.

This study aims to test whether the color of European sea bass (*Dicentrarchus labrax* L.) reared using an organic protocol is different from that resulting from a conventional approach.

MATERIALS AND METHODS

The experiment was carried out in an experimental facility (Costa *et al*., 1999) on the Lake of Sabaudia (LT, Italy), a coastal lagoon 100 km SE of Rome.

Besides different densities, sea bass in the organic protocol were fed with a special diet (EcoLife Pearl 864, 4.5 mm), while fish in the conventional protocol were supplied commercial feed (Ytelse M 664, 4.5 mm) of the same manufacturer (BioMar sas, Nersac, France). Basic ingredients, proximate composition and main energy characteristics are reported in Table1.

Table 1. Ingredients, proximate composition and energy characteristics of organic (EcoLife Pearl 864) and conventional (Ytelse M 664) sea bass feeds (from manufacturer's notice).

		organic	conv.			organic	conv.
proteins	%	46.0	44.0	total energy	MJ	20.0	22.5
lipids	%	15.0	20.0	digestible energy	MJ	17.0	19.7
carbohydrates	%	17.0	22.0	energy from proteins	%	58.0	51.0
ash	%	11.6	6.5	energy from fats	%	32.0	41.0
water	%	10.4	7.5	proteins/energy	g/MJ	24.3	21.7

EcoLife: fish meal, organic peas, organic soya cake, fish oil, minerals, vitamins

Ytelse: corn gluten, peas, soya cake, fish meal, rapeseed cake, fish oil, rapeseed oil, peanut cake, minerals, vitamins

The experiment started in March 2011, when a sample of unweaned fish was measured and photographed, and the differential feeding began (organic (O) *vs*. conventional (C)). In order to measure the fish color pattern, the sea bass were checked 4 times (from T_1 to T_4), and a total of 195 images of individual fish were validated. The number of images for each rearing treatment and time is reported in Table 2, together with the mean standard length (SL, cm). The increase in weight of the sea bass fed organic or conventional protocols were very similar, as well as their respective condition factors.

Table 2. Sampling dates, number of valid images, mean standard length (SL) and mean condition factor (CF) of sea bass reared under organic and conventional protocol.

	date	n. organic (O) mean SL±StD (cm)	n. conventional (C) mean SL±StD (cm)
T_0	25/03/2011	19 21.6±1.8	17 21.1±1.1
T_1	06/05/2011	14 22.6±1.3	13 22.9±1.3
T_2	17/06/2011	26 22.7±1.3	24 22.5±0.9
T_3	27/07/2011	21 23.9±1.2	16 24.1±1.4
T_4	14/09/2011	22 24.6±1.2	23 24.6±1.5

Color calibration and validation was carried out using a 32 color-patch ColorChecker (the GretagMacbeth ColorChecker 24 color-patches together with other 8 patches which better overcompensate the RGB color space; Costa *et al.*, 2013b) as reference standard. Matlab was used to perform the image calibration based on TPS-3D calibration (Menesatti *et al*., 2012). For each patch of the ColorChecker the spectral reflectance values from 400 to 700 nm was extracted using a portable integrated-sphere D50/2 spectrocolorimeter. Spectral reflectance values were converted in sRGB (Furusawa *et al.*, 2010) using Matlab OptProp.

After color calibration, a total number of 16 landmarks were digitized (Fig. 1) on each fish image, in order to allow the comparison of the entire body fish area. The first 12 landmarks were used to contour the region of interest (ROI) to be compared among samples. Following the landmarks configuration, the image RGB matrices were war-

ped through a geometric morphometry procedure (Costa *et al*., 2009; 2013a; Menesatti *et al*., 2010). This way, each pixel inside each ROI could be compared with the one in the same position of the other samples. For each individual, the 3 RGB values of the 45,075 pixels composing the ROI were decomposed in a single row (135,225 values).

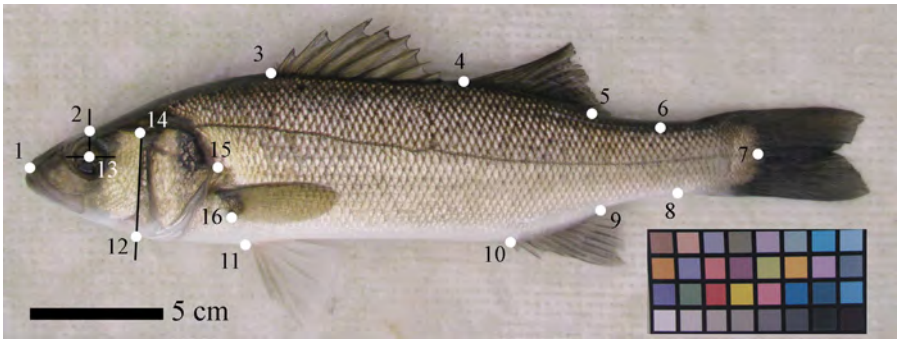

Figure 1. Landmarks (white dots) on sea bass, and the 32 color-patch ColourChecker.

The whole matrix (195 x 135,225 elements) representing the RGB color values inside the ROI of each fish was first analyzed with a 50-50 MANOVA procedure (Langsrud, 2002). A partial least square regression (PLS) was applied on the whole matrix in order to observe the relationship between color and size (SL) or condition index (CI = 100 * weight * length^{-3}). A partial least square discriminant analysis (PLSDA) was used to build a model discriminating between: *i.* T_0 *vs*. T_4 (O *vs*. C) (3 classes), *ii.* T_4 O *vs*. T_4 C (2 classes), *iii.* all the combinations between rearing treatments (O *vs*. C) and sampling times (from T_0 to T_4) (9 classes, considering T_0 O and T_0 C as the same class).

A dataset partitioning (75% to the model building, 25% for the testing procedure) was carried out by: 1) a partitioning algorithm that takes into account the variability in both X- and Y-spaces called sample set partitioning based on joint x-y distances (SPXY; Harrop Galvão *et al*., 2005) for PLS approach; 2) an extraction function based on distances and on the Kennard-Stone algorithm for PLSDA approach (Kennard & Stone, 1969).

The degree of estimation accuracy in quantitative prediction (PLS) must be inferred by the direct comparison between the measured and the estimated response variable, by calculating different parameters of the prediction efficiency: coefficient of correlation (r) between measured and predicted values, RMSE (root mean square error); SEP (standard error of prediction). The PLSDA analysis provides the percentage of correct classification of each class. This analysis expresses also the statistical estimates indicating the modeling efficiency arising from sensitivity and specificity parameters (Costa *et al*., 2008). RPD is the ratio between the standard deviation of the measured data and the RMSE, and was calculated on both the training and the validation set. The load of each pixel (X-block), in first latent vector (LV), was extracted (Costa *et al*., 2009) in order to determine the pixels contribution to the PLSDA classifications.

For each ROI the mean RGB values for each rearing treatments (O *vs*. C) and sampling times (from T_0 to T_4) (9 classes, considering T_0 O and T_0 C as the same class) were calculated. A dendrogram (single linkage) based on the mean Euclidean distances, between each rearing treatment (O *vs*. C) and sampling times (from T_0 to T_4) (9 classes, considering T_0 O and T_0 C as the same class), based on the 3 RGB values decomposed in a single row (135,225 values), was finally built.

RESULTS

The results of the 50-50 MANOVA are reported in Table 3. It is possible to observe that condition index, sampling time and their interaction all appear significant, using 32, 16 and 33 PCs, respectively.

Table 3. Results of the whole MANOVA. df: degrees of freedom. exVarSS: (Sum of SS for each response) / (Sum of total SS for each response). nPC: number of principal components used. nBuf: number of PCs used as buffer components. exVarPC: variance explained by nPC components. exVarBuf: variance explained by (nPC+nBuf) components. p-values: 50-50 MANOVA p-values.

source	df	exVarSS	nPC	nBuf	exVarPC	exVarBuf	p-value
rearing treatment (RT)	1	0.05526	32	75	0.504	0.810	>0.00001
sampling time (ST)	4	0.22829	16	83	0.501	0.818	>0.00001
RT*ST	4	0.05918	33	74	0.501	0.803	>0.00001
error	185	0.65967					

The summary of the PLS regression is reported in Table 4. Following the classification proposed by Viscarra-Rossel *et al*. (2007), the RPD_{RMSE} values of the testing procedure of both SL and condition index range from 1.0< RPD <1.4, indicating a poor prediction model, and, even if the RPD_{RMSE} values of the testing procedure are high, the models show a scarce relationship between color and SL or condition index. These results were confirmed also by the low r values of the testing procedure (0.69 and 0.76, respectively).

Table 4. Results of partial least squares (PLS) regression to relate color and SL or condition index. r= correlation coefficient; SEP= standard error of prediction; RMSE= root mean squares error; RPD_{RMSE}= ratio of percentage deviation for both model and testing sets.

	model		testing	
	SL	**condition index**	**SL**	**condition index**
n. latent vectors	8	4	8	4
r	0.9993	0.9617	0.6862	0.7569
SEP	0.059	0.059	1.591	0.199
RMSE	0.059	0.059	1.601	0.225
RPD_{RMSE}	27.175	3.651	1.296	1.304

The principal results of the PLSDA models are presented in Table 5 for: *i.* T_0 and T_4 (O *vs*. C) (T_0 *vs*. T_4), *ii.* T_4 O and T_4 C (T_4), *iii.* all the combinations between rearing treatments (O *vs*. C) and sampling times (from T_0 to T_4) (Total). The 3 models correctly classify all samples within their own classes, both in model and in testing datasets (only one individual T_3 O in the Total testing dataset was misclassified as T_4 C). Scores of

the Total testing samples on first 2 LVs are reported in Figure 2; note how the organic fishes (filled symbols) were more similar to the T_0 (i.e., wild; crosses) samples than to the conventional ones (empty symbols).

Table 5. Results of partial least squares discriminant analysis (PLSDA) for: T_0 vs. T_4 (O *vs.* C) (T_0 *vs.* T_4), T_4 O *vs.* T_4 C (T_4) and all the combinations between rearing treatments (O vs. C) and sampling times (from T_0 to T_4) (Total).

	T_0 *vs.* T_4	T_4	total
N	81	45	195
n. of latent vectors	3	3	7
n. of classes	3	2	9
Sensitivity	1	1	1
Specificity	1	1	0.98
% probability of random assignment of an individual	33.3	50.0	11.1
% correct classification in the model building (75%)	100	100	100
% correct classification in the testing procedure (25%)	100	100	97.8

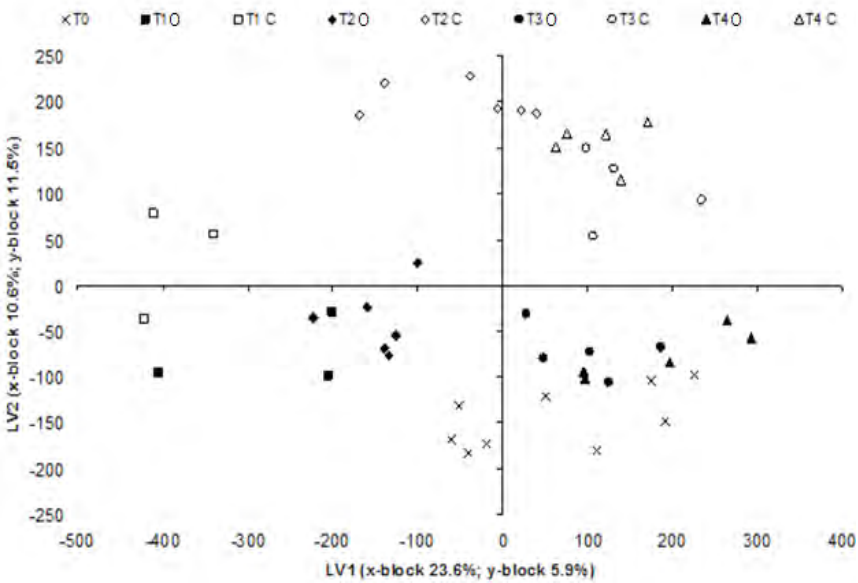

Figure 2. PLSDA Total testing dataset: scores of the samples belonging to each group on the first 2 latent vectors.

In Fig. 3, a dendrogram (single linkage) of the mean Euclidean distances, between each rearing treatment (O *vs.* C) and sampling time (from T_0 to T_4) (9 classes, considering T_0 O and T_0 C as the same class), based on the 3 RGB values decomposed in a single row (135,225 values) together with the relative mean RGB values within each ROI, is reported. It is possible to observe that the organic samples at T_2, T_3 and T_4 cluster together with T_0 (wild), while both samples at T_1 were much more distant; note also

how the head region is darker in the reconstructed representations of conventional fish (especially at T_2, T_3 and T_4).

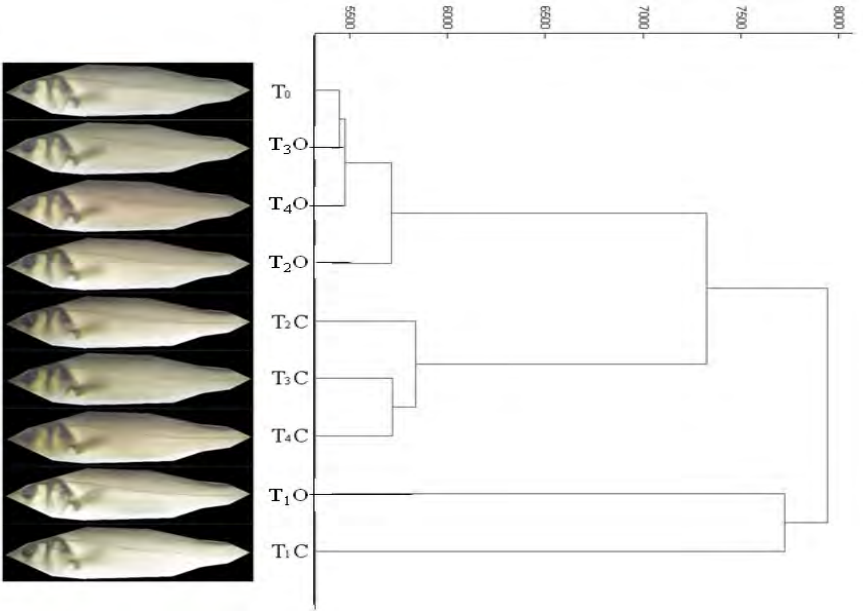

Figure 3. Dendrogram (single linkage) of the mean Euclidean distances between each rearing treatment (O *vs*. C) and sampling time (from T_0 to T_4), based on the RGB values decomposed in a single row, together with relative mean RGB values within each ROI.

DISCUSSION AND CONCLUSIONS

The preceding outcomes show that the two batches of fishes raised in different environmental conditions and fed different artificial diets, in the specific case following the organic vs. the conventional protocols, can be set apart also through their morphological characteristics, using their color appearance: moreover, the longer the time spent under the different regimens, the stronger the evidence of this outcome.

The body part that displays the greatest difference between the two groups is the head, which is of lighter color in fishes raised organically. Besides that, wild fishes are lighter than those arising from conventional aquaculture, at least in the present situation; nevertheless, data are not sufficient to generalize the similarity of appearance of natural and organic individuals.

It should be noted that the first samples under treatment (T_1), while being already distinct between themselves, are even more different from the following ones (T_2 to T_4), probably because the young fishes were still "weaning" (adapting to the artificial feeds) and thus presenting sub-optimal body conditions.

The present results are preliminary and apply only to the current data - which are limited in number, time and space - and therefore cannot be generalized to whole organic vs. conventional aquaculture. They may suggest a morphological difference between organic and conventional fishes, but in fact they prove only that the two specific and

peculiar farming conditions under study generated measurable differences. Those consequences are promising, but further experiments are required, longer in time, spread on many farms in different locations, with more batches of various origins and/or hatcheries, employing greater sample numerosity, and maybe other species. In fact, a similar approach may allow: to verify the coherence and stability of the measurements; to identify whether any specific parameter (e.g., origin, breeding density, feed, water quality, season, etc.) has dominant influence in determining the body color; to transfer the results obtained on the sea bass to other aquaculture species; to extend the methodology to the control of other quality "certifications" (e.g., besides the "organic aquaculture" label, the "protected geographical indication" IGP, the "controlled denomination of origin" DOP, the "sustainable agriculture" PAS, and so on).

In conclusion, a non-invasive colorimetric analysis, carried out with geometric morphology studies, can be used to discriminate sea basses arising from different, complex, raising protocols, and in this specific case to certify the fish as organically grown.

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REFERENCES

Bianchini M.L., Casadei S., Ingle E., Lombardi F. (1994): Competition effect on the survival of seabass fry without functional swim bladder. J. Ecobiol. 6, 1-4.

Bianchini M.L., Muzzini V., Pigliarino E. (2010): Trout hamburgers: a sustainable pipeline from aquaculture to community catering. Int. Aquat. Res. 2, 193-198.

Bianchini M.L., Palmegiano G.B. (1994): L'importanza delle specie "minori" nell'acquacoltura del bacino mediterraneo. Biol. Mar. Medit. 1: 445- 446.

Cataudella S., Mazzola A., Angle G., Boglione C., Crosetti D., Defrancesco E., Galeotti M., Orban E., Rambaldi E., Rampacci M., Russiello M., Ugolini R. (2001): Verso l'acquacoltura biologica? un programma Uniprom al servizio dei consumatori e dei produttori. Uniprom (Roma), 198 pp.

Costa C., Aguzzi J., Menesatti P., Antonucci F., Rimatori V., Mattoccia M. (2008): Shape analysis of different populations of clams in relation to their geographical structure. J. Zool. 276, 71-80.

Costa C., Angelini C., Scardi M., Menesatti P., Utzeri C. (2009): Using image analysis on the ventral colour pattern in *Salamandrina perspicillata* (Savi, 1821) (Amphibia, Salamandridae) to discriminate among populations. Biol. J. Linnean Soc. 96, 35-43.

Costa C., Antonucci F., Boglione C., Menesatti P., Vandeputte M., Chatain B. (2013a): Automated sorting for size, sex and skeletal anomalies of cultured seabass using external shape analysis. Aquac. Engineer. 52, 58-64.

Costa C., Antonucci F., Menesatti P., Pallottino F., Boglione C., Cataudella S. (2013b): An advanced colour calibration method for fish freshness assessment: a comparison between standard and passive refrigeration modalities. Food Bioprocess Technol., in press. DOI 10.1007/s11947-011-0773-6

Costa C., Bianchini M.L., Rambaldi E. (1999): L'Istituto di Idrobiologia e Acquacoltura "G. Brunelli". Notiz. SIBM 36, 93-94.

Costa C., Minervini R., Bianchini M.L. (1990): Thirty-five years of management in the coastal lagoon of Sabaudia, Italy. In: "Management of freshwater fisheries". Pudoc (Wageningen), 270-283.

Furusawa K., Suehara K.I., Kameoka T., Hashimoto A. (2010): Color appearance evaluation of agricultural products image based on spectral information of lighting. SICE Ann. Conf., Taipei (Taiwan) 18-21/08/2010.

Harrop Galvão R.K., Araujo M.C.U., José G.E., Coelho Pontes M.J., Silva E.C., Bezerra Saldanha T.C. (2005): A method for calibration and validation subset partitioning. Talanta 67, 736-740.

International Federation of Organic Agriculture Movements EU Group (2010): Acquacoltura biologica - regolamenti (CE) 834/2007, (CE) 889/2008, (CE) 710/2009: storia, valutazione, interpretazione. Dossier IFOAM, 40 pp.

Kennard R.W., Stone L.A. (1969): Computer aided design of experiments. Technometrics 11, 137-148.

Langsrud Ø. (2002): 50-50 multivariate analysis of variance for collinear responses. The Statistician 51, 305-317.

Menesatti P., Costa C., Aguzzi J. (2010): Quality evaluation of fish by hyperspectral imaging. In: Sun D.W. (ed.) "Hyperspectral imaging for food quality: analysis and control". Acad. Press (London), 273-294.

Menesatti P., Angelini C., Pallottino F., Antonucci F., Aguzzi J., Costa C. (2012): RGB color calibration for quantitative image analysis: the "3D Thin-Plate Spline" warping approach. Sensors 12, 7063-7079.

Viscarra-Rossel R.A., Taylor H.J., McBratney A.B. (2007): Multivariate calibration of hyperspectral gamma-ray energy spectra for proximal soil sensing. Eur. J. Soil Sci. 58, 343-353.